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The HIV-1 Tat Nuclear Localization Sequence Confers Novel Nuclear Import Properties*

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The different classes of conventional nuclear localization sequences (NLSs) resemble one another in that NLS-dependent nuclear protein import is energy-dependent and mediated by the cytosolic NLS-binding importin/karyopherin subunits and monomeric GTP-binding protein Ran/TC4. Based on analysis of the nuclear import kinetics mediated by the NLS of the human immunodeficiency virus accessory protein Tat using *in vivo* and *in vitro* nuclear transport assays and confocal laser scanning microscopy, we report a novel nuclear import pathway. We demonstrate that the Tat-NLS, not recognized by importin 58/97 subunits as shown using an enzyme-linked immunosorbent assay-based binding assay, is sufficient to target the 476-kDa heterologous β-galactosidase protein to the nucleus in ATP-dependent but cytosolic factor-independent fashion. Excess SV40 large tumor antigen (T-ag) NLS-containing peptide had no significant effect on the nuclear import kinetics implying that the Tat-NLS was able to confer nuclear accumulation through a pathway distinct from conventional NLS-dependent pathways. Nucleoplasmic accumulation of the Tat-NLS-β-galactosidase fusion protein, in contrast to that of a T-ag-NLS-containing fusion protein, also occurred in the absence of an intact nuclear envelope, implying that the Tat-NLS conferred binding to nuclear components. This is in stark contrast to known NLSs such as those of T-ag which confer nuclear entry rather than retention. Significantly, the ability to accumulate in the nucleus in the absence of an intact nuclear envelope was blocked in the absence of ATP, as well as by nonhydrolyzable ATP and GTP analogs, demonstrating that ATP is required to effect release from a complex with insoluble cytoplasmic components. Taken together, the results demonstrate that, dependent on ATP for release from cytoplasmic retention, the Tat-NLS is able to confer nuclear entry and binding to nuclear components. These unique properties indicate that Tat accumulates in the nucleus through a novel import pathway.

To enter the eukaryotic cell nucleus, proteins larger than 45 kDa require targeting signals called nuclear localization sequences (NLSs)[1] defined as the sequences sufficient and necessary for nuclear localization of their respective proteins (1, 2). NLSs appear to fall into several classes, including those homologous to the NLS of the simian virus SV40 large tumor-antigen (T-ag) consisting of a single stretch of basic residues (1–3), those termed bipartite NLSs comprising two clusters of basic amino acids separated by a spacer of 10–12 amino acids (1, 4) and those resembling the NLS of the yeast homeodomain protein Mata2 (NKIPIKDLLNPQ[13] (5)). All of these types of NLS are similar in terms of the transport process and the cytosolic factors mediating it (see Refs. 1, 2, and 6), whereby NLS-containing proteins are initially bound by a heterodimer consisting of proteins of about 60 and 95 kDa, variously named importin α/β (7), importin 58/97 (8), and karyopherin α/β (9). The smaller importin/karyopherin subunit binds the NLS specifically, whereas the larger subunit both enhances the affinity of the complex for the NLS (6, 9–11) and mediates the docking of the cargo-carrier complex to the nuclear pore complex (NPC). The second, energy-dependent step involves transfer of the cargo-carrier complex to the nucleoplasmic side and requires GTPase activity on the part of the monomeric GTP-binding protein/GTPase Ran/TC4 and other factors such as NTF2 (see Refs. 1, 2, and 12–14).

While the conventional NLSs mentioned above appear to be recognized by importin/karyopherin and transported to the nucleus as outlined above, recent studies have revealed two novel nuclear protein import pathways which are mediated by quite distinct targeting signals and do not appear to involve the importin 58/97 complex (15–17). Nuclear import of the nuclear-cytoplasmic shuttling hnRNP protein A1 is mediated by an importin-97-homolog transportin (karyopherin β2), which recognizes the A1 "M9" NLS but does not interact with the more conventional NLSs referred to above (15, 16). In contrast, nuclear import of the shuttling hnRNP K protein through the NPC conferred by the "KNS" NLS-sequence does not appear to require a soluble cytosolic receptor or Ran (17). Conventional NLSs, as well as the M9 and KNS NLSs, do not mediate nuclear accumulation by conferring binding to nuclear components, but function exclusively as nuclear entry signals (1, 6, 15).

We have been interested for some time in the nuclear import of viral proteins (6, 11), and in particular the human immunodeficiency virus type 1 (HIV-1) Tat protein, which is a potent activator of viral gene expression and replication (see Ref. 18). Tat accumulates predominantly in the nucleus and nucleolus (19–21) through possession of an amino-terminal stretch of basic amino acid residues purported to be the NLS

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[1] The abbreviations used are: NLS, nuclear localization sequence; T-ag, simian virus SV40 large tumor-antigen; NPC, nuclear pore complex; Tat, human immunodeficiency virus type 1 Tat protein; K_D, apparent dissociation constant; CHAPS, 3-[(3-cholamidopropyl)dimethylammoniol-1-propanesulfonate; GST, glutathione *S*-transferase; CLSM, confocal laser scanning microscopy; HTC, hepatoma tissue culture; ELISA, enzyme-linked immunosorbent assay; AMP-PNP, adenylyl imidodiphosphate; GTP-γS, guanosine 5′-3-*O*-(thio)triphosphate; hnRNP, heterogeneous ribonucleoprotein.

(GRKKRRQRRRAP[59], single-letter amino acid code; basic residues highlighted in bold type) (22, 23), which is highly conserved among HIV-*I* isolates. The Tat-NLS resembles similar highly basic amino-terminal sequences of the HIV-1 Rev (RQARRNRRRRWRERQRQ[81] (24)) and the HTLV-1 (human T-cell leukemia virus) Rex (MPKTRRRPRRSQRKRPPTP[119]) proteins, both of which have been shown to constitute functional NLSs (24–27).

To gain insight into Tat targeting function as a possible paradigm of this class of viral targeting signal, this study examines the nuclear import kinetics of a β-galactosidase fusion protein carrying Tat amino acids 48–59 *in vivo* and *in vitro* at the single cell level, comparing results to those for fusion proteins carrying a classical NLS, that of T-ag (3). We find that the Tat-NLS, in contrast to the classical T-ag-NLS, confers nuclear accumulation through an import pathway which appears to require ATP but not cytosolic factors such as importin. Experiments using cells in which the nuclear envelope was permeabilized with CHAPS indicate that Tat fusion proteins can bind to both insoluble cytoplasmic and nuclear factors and that ATP is required to effect release from cytoplasmic retention and relocation to the nucleus. In contrast, the T-ag fusion protein binds neither cytosolic nor nuclear factors under the same conditions. We conclude that the Tat-NLS is able to target β-galactosidase to the nucleus through a novel import pathway.

MATERIALS AND METHODS

Chemicals and Reagents—The detergent CHAPS was from Boehringer Mannheim and AMP-PNP from Calbiochem. The bacterial strains for karyopherin α (Kap60) and β (Kap95) fusion protein expression (9) were provided by Michael Rexach. Other reagents were from the sources previously described (6, 11, 28–31).

Cell Culture—Cells of the HTC rat hepatoma tissue culture (a derivative of Morris hepatoma 7288C) line were cultured as described previously (28, 29).

β-Galactosidase Fusion Proteins—The plasmid expressing the Tat-NLS-β-galactosidase fusion protein Tat-NLS-β-Gal was derived by oligonucleotide insertion into the *Sma*I restriction endonuclease site of the plasmid vector pPR2 (29). The resultant fusion protein comprises Tat amino acids 48–59 (GRKKRRQRRRAP[59]) fused amino-terminal to the *Escherichia coli* β-galactosidase enzyme sequence (amino acids 9–1023). The T-ag β-galactosidase fusion protein (T-ag-CeN-β-Gal) used in the comparative studies contains T-ag amino acids 111–135, including the CeN motif (comprising protein kinase CK2 and gdk phosphorylation sites and the NLS) fused amino-terminal to β-galactosidase amino acids 9–1023 (28, 29). 1 mM isopropyl-β-thiogalactoside was used to induce expression of fusion proteins in *E. coli*. They were purified by affinity chromatography and labeled using the sulfhydryl labeling reagent 5-iodoacetamidofluorescein (Molecular Probes) as described (29).

Nuclear Import Kinetics—Nuclear import kinetics at the single cell level were measured using either microinjected (*in vivo*) or mechanically perforated (*in vitro*) HTC cells in conjunction with confocal laser scanning microscopy (CLSM) (6, 11, 28–31). In the case of microinjection, HTC cells were fused with polyethylene glycol about 1 h prior to microinjection to produce polykaryons (6, 11, 28, 29, 31). Reticulocyte lysate (Promega) was used as the source of cytosol for the *in vitro* assay (6, 28, 30, 31). Image analysis of CLSM files using the NIH Image public domain software and curve-fitting were performed as described (6, 11, 30, 31).

In *in vitro* experiments where the ATP dependence of transport was tested, apyrase pretreatment was used to hydrolyze endogenous ATP in both cytosol (10 min at room temperature with 800 units/ml) and perforated cells (15 min at 37 °C with 0.2 unit/ml) (6, 30, 32), and transport assays were performed in the absence of the ATP regenerating system (28, 30) which was otherwise used. Where the dependence on the GTP-binding protein Ran was tested, cytosolic extract was treated with 860 μM GTPγS (nonhydrolyzable GTP analog) for 5 min at room temperature, prior to use in the *in vitro* assay (final concentration of 300 μM) (6, 12, 13, 30). The ability of GTP to substitute for ATP in the transport assay was assessed by replacing the ATP-regenerating system with 2 mM GTP/2 mM GDP.

In competition experiments, peptides P101 (CGPGSDDEAAADAQHAAPPKKKRKVGY, including T-ag amino acids 111–132) and P101T (identical to P101, but containing the NLS-inactivating Lys^{128} to Thr substitution) (3, 11, 29, 33) were used at final molar concentrations 200-fold those of the Tat and T-ag fusion proteins (4.2×10^{-7} M). Nuclear accumulation was also examined *in vitro* in the presence of 1% glycerol and 0.025% CHAPS which results in permeabilization of the nuclear envelope; accumulation under these conditions results solely from binding to nuclear components such as lamins, chromatin etc. (6).

ELISA-based Binding Assay—An ELISA-based binding assay (6, 11, 31) was used to examine the binding affinity between importin subunits (mouse importin 58 and 97 glutathione *S*-transferase (GST)- fusion proteins, expressed as described (8, 11)) and Tat or T-ag fusion proteins. This involved coating 96-well microtiter plates with β-galactosidase fusion proteins, hybridization with increasing concentrations of importin subunits, and detection of bound importin-GST using goat anti-GST primary, and alkaline phosphatase-coupled rabbit anti-goat secondary antibodies, and the substrate *p*-nitrophenyl phosphate (6, 11). Absorbance measurements were performed over 90 min using a plate reader (Molecular Devices), and values were corrected by subtracting absorbance both at 0 min and in wells incubated without importin (6, 11, 31). To quantitate importin binding specifically to the NLSs, quantitation was performed in identical fashion for β-galactosidase itself, and the values were subtracted from those for the respective fusion proteins (6, 11, 31). Fusion proteins were also subjected to a parallel β-galactosidase ELISA (see Refs. 6, 11, and 31) to correct for any differences in coating efficiencies and enable a true estimate of bound importin (6, 11). Measurements for the NLS binding affinity of the karyopherin subunits were performed in identical fashion to those for importin 58/97 using GST-fusion proteins expressed in *E. coli* (9).

RESULTS AND DISCUSSION

The NLS of HIV-1 Tat Is Capable of Targeting a Heterologous Protein to the Nucleus—To examine the ability of the HIV-1 Tat basic region to target a large (476-kDa) heterologous protein (β-galactosidase from *E. coli*) to the nucleus, a plasmid was derived expressing fusion protein Tat-NLS-β-Gal containing Tat amino acids 48–59 (see "Materials and Methods") fused amino-terminal to β-galactosidase amino acids 9–1023. Its nuclear import kinetics were measured *in vivo* and *in vitro* using microinjected cells of the HTC line (6, 11, 28, 29, 31) and mechanically perforated HTC cells (6, 28, 30, 31), respectively, and compared with those for a fusion protein (T-ag-CeN-β-Gal) carrying the T-ag NLS and β-galactosidase itself. The Tat-NLS targeted β-galactosidase to the nucleus in both assay systems (Figs. 1 and 2), Tat-NLS-β-Gal accumulating maximally to levels about 2-fold those in the cytoplasm (Figs. 1*B* and 2*B*; Table I). The extent of maximal accumulation of Tat-NLS-β-Gal was markedly lower than that of T-ag-CeN-β-Gal which attained levels over 5-fold those in the cytoplasm (Table I). The transport rate of Tat-NLS-β-Gal *in vivo* was markedly higher (rate constant (k) of 0.3) than that of T-ag-CeN-β-Gal (k = 0.125) (see Table I). As observed previously (28, 29), β-galactosidase was completely excluded from the nucleus both *in vivo* and *in vitro* ($Fn/c_{max} < 0.65$, Figs. 1*B* and 2*B*; Table I). Although Tat nucleolar localization has been reported using transfection systems (19–21), we did not observe anything other than nucleoplasmic accumulation (Figs. 1 and 2 and see below). That the lack of nucleolar accumulation is unlikely to be an artifact of the systems used is indicated by our previous studies examining nucleolar import of other proteins *in vitro* (30),[2] and we conclude that Tat amino acids 48–59 do not confer nucleolar localization.

Independence of Nuclear Uptake Conferred by the Tat-NLS on Cytosolic Factors—Conventional NLS-mediated nuclear protein import *in vitro* is dependent on energy (6, 28, 30, 32) and the addition of exogenous cytosol (6–10, 28, 30, 32). The latter supplies the importin 58/97 NLS-binding/NPC-docking dimer (7–9) as well as the GTPase Ran (12, 13) and interacting proteins (see Refs. 1, 2, and 14), which are essential for nuclear

[2] A. Efthymiadis, L. J. Briggs, and D. A. Jans, unpublished results.

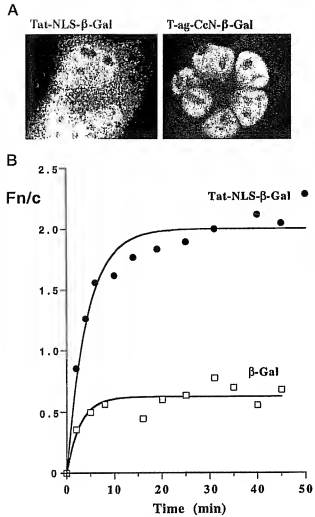

FIG. 1. **Nuclear import of fusion protein Tat-NLS-β-Gal *in vivo*.** *A*, CLSM images are shown for polykaryons 30 min after microinjection; results are compared with those for the T-ag NLS-containing fusion protein T-ag-CcN-β-Gal (*right panel*, see Table I for quantitative data). *B*, nuclear import kinetics. Measurements were performed as described under "Materials and Methods" (19, 23) and represent a single typical experiment, where each point represents the average of 10–12 separate measurements for each of nuclear, cytoplasmic, and background (autofluorescence) fluorescence. Data were fitted for the function $Fn/c(t) = Fn/c_{max} \times (1 - e^{-kt})$, where Fn/c is defined as the ratio of nuclear to cytoplasmic fluorescence after the subtraction of fluorescence due to autofluorescence (19, 21, 23); collated data are presented in Table I. Results for Tat-NLS-β-Gal are compared with those for β-galactosidase (*β-Gal*).

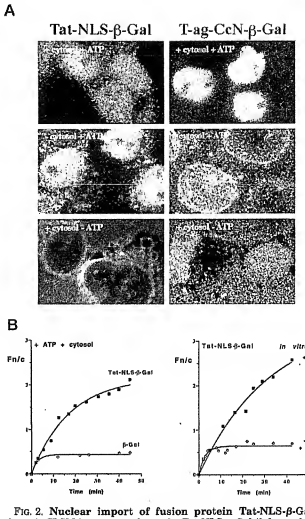

FIG. 2. **Nuclear import of fusion protein Tat-NLS-β-Gal *in vitro*.** *A*, CLSM images are shown for Tat-NLS-β-Gal (*left panels*) and T-ag-CcN-β-Gal (*right panels*, see Table I for quantitative data) in the presence and absence of either exogenously added cytosol and/or an ATP-regenerating system as indicated after 30 min at room temperature (see "Materials and Methods"). *B*, nuclear import kinetics. Experiments were carried out in the absence and presence of exogenous cytosol and/or an ATP-regenerating system as indicated; measurements and curve-fitting were performed as described in the legend to Fig. 1*B* and represent the average of at least two separate experiments, where each point represents the average of up to 10 separate measurements for each of Fn and Fc, respectively, with autofluorescence subtracted. Results for Tat-NLS-β-Gal in the presence of cytosol/ATP are compared with those for β-galactosidase (*β-Gal*) (*left panel*).

accumulation. Analogously, M9-mediated nuclear import of hnRNP A1 requires the cytosolic NLS-binding transportin protein and Ran (15, 16, 34). Nuclear import of Tat-NLS-β-Gal was found to be dependent on ATP but not on exogenous cytosol, in contrast to that of T-ag-CcN-β-Gal which required both ATP and cytosol (Fig. 2; Table I). Interestingly, accumulation of Tat-NLS-β-Gal in the presence of ATP but without cytosol was 50% increased compared with that in the presence of cytosol, implying that the latter inhibited transport. The nonhydrolyzable GTP analog GTPγS was able to inhibit nuclear accumulation of both Tat-NLS-β-Gal and T-ag-CcN-β-Gal in the presence of the ATP regenerating system; the nonhydrolyzable ATP analog AMP-PNP similarly inhibited nuclear transport (Table I). Nuclear accumulation of Tat fusion proteins thus appears to be an active process. GTP/GDP could not substitute for ATP to permit nuclear accumulation (see Table I), which, together with its cytosolic independence, implies that a role for Ran in Tat-NLS-mediated nuclear import is unlikely (see Refs. 13, 35, and 36). That other GTP-binding proteins appear to play a role in nuclear protein import (see Refs. 37 and 38) may constitute the basis of the inhibition of Tat-NLS-mediated nuclear import by GTPγS.

To confirm that Tat-NLS-β-Gal accumulates in the nucleus through a pathway distinct from that used by conventional NLSs, we carried out competition experiments *in vitro* using T-ag-NLS-containing peptides (33). A 200-fold excess of the wild type T-ag NLS-containing peptide P101 (see "Materials and Methods") essentially abolished nuclear accumulation of T-ag-CcN-β-Gal, the specificity of this effect being demonstrated by the fact that the same concentration of the NLS-deficient (Thr^{128}) peptide P101T had no effect (Fig. 3, *right panel*). In contrast, nuclear accumulation of Tat-NLS-β-Gal was completely unaffected by either peptide (Fig. 3, *left panel*), supporting the idea that the Tat-NLS conferred nuclear trans-

TABLE I
Nuclear import kinetics of Tat-NLS-β-Gal compared with those of T-ag-CcN-β-Gal and β-galactosidase

Protein/conditions	Nuclear import parameter[a]		
	Fn/c_{max}	k (10^{-3})	n
A. *In vivo* (microinjected cells)			
Tat-NLS-β-Gal	2.05 ± 0.20	303 ± 99	3
T-ag-CcN-β-Gal[b]	7.47 ± 1.10	125 ± 19	4
β-Galactosidase	0.63 ± 0.03[c]	ND[d]	1
B. *In vitro* (mechanically perforated cells)			
Tat-NLS-β-Gal			
+ ATP + cytosol	2.20 ± 0.24	62 ± 5	11
+ ATP − cytosol	3.04 ± 0.11	26 ± 3	4
+ ATP − cytosol + AMP-PNP	1.70 ± 0.12[e]	6 ± 1	1
− ATP[e] + cytosol	0.63 ± 0.03[e]	ND[d]	1
− ATP[f] − cytosol	0.71 ± 0.06	ND[d]	2
− ATP[f] − cytosol + GDP/GTP	0.42 ± 0.07	ND[d]	2
+ ATP + cytosol + GTPγS	0.96 ± 0.13[e]	ND[d]	1
T-ag-CcN-β-Gal[b]			
+ ATP + cytosol	5.06 ± 0.75	56 ± 27	4
+ ATP − cytosol	1.10 ± 0.04	ND[d]	4
−ATP[e] + cytosol	1.30 ± 0.10[e]	ND[d]	1
− ATP[f] − cytosol	0.99 ± 0.03	ND[d]	4
+ ATP + cytosol + GTPγS	2.23 ± 0.27[e]	ND[d]	1
β-galactosidase			
+ ATP + cytosol	0.46 ± 0.002	ND[d]	2
70 kDa dextran			
+ ATP + cytosol	0.22 ± 0.03	ND[d]	5
C. *In vitro* (mechanically perforated cells) + CHAPS[g]			
Tat-NLS-β-Gal			
+ ATP + cytosol	2.40 ± 0.13	ND[d]	3
+ ATP − cytosol	2.18 ± 0.16	ND[d]	4
+ ATP − cytosol + AMP-PNP	1.35 ± 0.19[e]	ND[d]	1
+ ATP − cytosol + GTPγS	1.55 ± 0.05[e]	ND[d]	1
− ATP[e] + cytosol	1.20 ± 0.11	ND[d]	2
− ATP[f] − cytosol	0.38 ± 0.08	ND[d]	4
− ATP[f] − cytosol + GDP/GTP	0.41 ± 0.03	ND[d]	2
T-ag-CcN-β-Gal			
+ ATP + cytosol	1.26 ± 0.06[e]	ND[d]	1
− ATP[f] − cytosol	1.20 ± 0.15	ND[d]	2
70 kDa dextran			
+ ATP + cytosol	0.98 ± 0.06	ND[d]	1
− ATP[f] − cytosol	0.89 ± 0.09[e]	ND[d]	1

[a] Raw data (see Figs. 1B and 2B and data not shown) were fitted for the function $Fn/c(t) = Fn/c_{max} \times (1 - e^{-kt})$ (6, 11, 28–31), where Fn/c_{max} is the maximal level of accumulation at steady state in the nucleus, and t is time in minutes. An Fn/c_{max} of 1.0 indicates equilibration between nucleus and cytoplasm, with values below 1 indicating exclusion from the nucleus. The S.E. is indicated.

[b] The T-ag β-galactosidase fusion protein (described in Ref. 29) contains the NLS together with the regulating phosphorylation sites, known as the CcN motif (28).

[c] S.E. from curve fit.

[d] ND, not able to be determined.

[e] Apyrase pretreatment was used, and the ATP-regenerating system was omitted (see "Materials and Methods").

[f] Cytosol and the ATP-regenerating system were omitted (no apyrase pretreatment).

[g] Nuclear accumulation in the presence of the detergent CHAPS (see "Materials and Methods"); accumulation indicates binding to nuclear components (6).

port through a pathway distinct from that conferred by the T-ag-NLS.

Nuclear Accumulation of Tat-NLS-β-Gal in the Absence of an Intact Nuclear Envelope—Detergents such as CHAPS can be used to perforate the nuclear envelope to enable molecules to diffuse freely between cytoplasm and nucleoplasm; nuclear accumulation under these conditions occurs through binding to nuclear components (6). As observed previously (6), T-ag-CcN-β-Gal did not accumulate in the nucleus under these conditions (Fig. 4, *top right panel*; Table I), instead showing equilibration between nuclear and cytoplasmic compartments in the absence of a barrier to diffusion. In contrast, Tat-NLS-β-Gal accumulated quite well in the absence of cytosol, but only in the presence of ATP (Fig. 4, *left panels*; Table I). Surprisingly, despite the absence of a barrier to diffusion, Tat-NLS-β-Gal exhibited quite marked nuclear exclusion due to cytoplasmic association in the absence of ATP (Fig. 4, *bottom right panel*; Table I). GTP/GDP could not substitute for ATP in terms of facilitating nuclear accumulation (Table I), while both GTP and ATP analogs inhibited accumulation in the presence of the ATP regenerating system. The results indicate that, in contrast to the conventional T-ag-NLS, which, although not preventing nuclear entry in the absence of an intact nuclear envelope, does not confer nuclear accumulation (see also Ref. 6), the Tat-NLS is able to confer nuclear accumulation in the absence of an intact nuclear envelope. In the absence of ATP hydrolysis, Tat-NLS-β-Gal appears to exhibit high affinity for an insoluble cytoplasmic factor, while in its presence, it can accumulate in the nucleus through binding to nucleoplasmic components. Consistent with these conclusions, interaction of the complete Tat molecule with either cytoplasmic or nuclear components, varying according to the phase of HIV-1 infection, has been reported (38).

Lack of Recognition of the Tat-NLS by the Conventional NLS-binding Importin 58/97 Dimer—To test directly whether importin subunits could recognize the Tat-NLS, we used a previously established, specific ELISA-based binding assay (6, 11, see "Materials and Methods"). Tat-NLS-β-Gal and T-ag-CcN-β-Gal fusion proteins were coated onto microtiter plates, incubated with increasing amounts of importin 58-GST, importin 97-GST, or importin 58/97-GST complex, and binding was then quantitated using antibodies specific to GST and an alkaline phosphatase-labeled secondary antibody as previously described (11). Comparable with previous measurements (6, 11, 31) the apparent dissociation constant (K_D) of T-ag-CcN-β-Gal for importin 58 and 58/97 was 45 and 9.6 nM, respectively. In contrast, Tat-NLS-β-Gal exhibited no detectable binding of either 58 or 58/97 above that of β-galactosidase alone. No binding by importin 97-GST to either T-ag-CcN-β-Gal or Tat-NLS-β-Gal could be detected. Similar results were obtained for the karyopherin subunits (9). The lack of binding of Tat-NLS-β-Gal by importin/karyopherin subunits was thus consistent with our *in vitro* transport results indicating that nuclear accumulation of the Tat fusion protein does not require cytosolic factors.

Mechanism of Tat-NLS-conferred Nuclear Accumulation—The observation that Tat-NLS-β-Gal may have high affinity for an insoluble cytoplasmic factor in the absence of ATP inspired us to test whether cytoplasmic retention could be overcome by increasing the relative concentration of Tat-NLS-β-Gal. Assays were accordingly performed using up to 18-fold higher concentrations of Tat-NLS-β-Gal (where the amount of labeled protein was kept constant, and final Tat-NLS-β-Gal concentration was adjusted through the addition of unlabeled protein) than the standard concentration (4×10^{-7} M) used in the *in vitro* assay. Measurements in the presence of ATP showed a small (~25%) increase in maximal accumulation (Fn/c_{max} of 3.8) at 1.6×10^{-6} compared with at 4×10^{-7} M (Fn/c_{max} of 2.9), but a significant reduction at higher concentrations (Fn/c_{max} of 1.6 at 7.2×10^{-6} M). This implied that rather than a cytoplasmic retention factor, some other transport component is limiting, *e.g.* the number of sites for Tat-NLS binding within the nucleus may be titratable (see also below).

Similar experiments were performed in the presence of CHAPS, where increasing the concentration in the presence of ATP had no effect on cytoplasmic retention; even at 7.2×10^{-6} M Fn/c_{max} was only 1.3. Increasing the concentration of Tat-NLS-β-Gal in the absence of ATP did not effect any release from cytoplasmic retention, maximal accumulation at 7.2×10^{-6} M being lower (Fn/c_{max} of 0.36) than that at 4.2×10^{-7} M

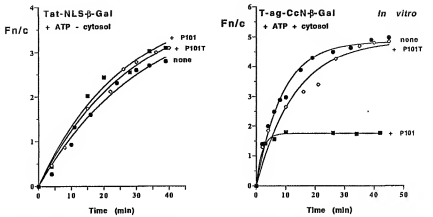

FIG. 3. **Nuclear uptake of Tat-NLS-β-Gal *in vitro* in the absence and presence of T-ag NLS-containing peptides.** Experiments were performed in the absence and presence of a 200-fold excess of peptides P101 (including T-ag NLS and flanking region) or P101T (the NLS-mutant version of P101) and an ATP-regenerating system. Measurements were performed as described in the legend to Fig. 2B and represent results from a single typical experiment. The import rates for Tat-NLS-β-Gal were 31 ± 1, 39 ± 2, and 36 ± 1 × 10^{-3} in the presence of no peptide, peptides P101 and P101T, respectively. Results for Tat-NLS-β-Gal are compared with those for T-ag-CcN-β-Gal (*right panel*); in the presence of P101T, the rate of import was reduced by 32% compared with in the absence of peptide.

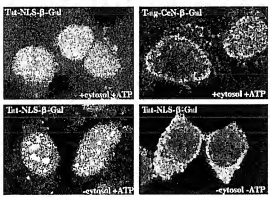

FIG. 4. **Nuclear accumulation of Tat-NLS-β-Gal *in vitro* in the presence of the nuclear envelope-permeabilizing detergent CHAPS is dependent on ATP.** Experiments were performed as described in the legend to Fig. 2B in the presence of 0.025% CHAPS in the absence or presence of cytosol and/or an ATP-regenerating system as indicated. Nuclear accumulation indicates binding to nuclear components (6). Results are compared with those for T-ag-CcN-β-Gal (see Table I for collated quantitative data).

(Fn/c_{max} of 0.54). The hypothesis that the Tat-NLS confers binding to a titratable cytoplasmic retention factor is thus inconsistent with the experimental observations, the fact that increasing the concentration of Tat-NLS-β-Gal above a certain threshold reduces the maximal level of accumulation in the presence of CHAPS being consistent with nuclear binding sites for Tat being limiting.

Novel Nuclear Import Pathway Conferred by the Tat-NLS—The results above indicate that the Tat-NLS is capable of targeting a large heterologous protein to the nucleus through a pathway which is dependent on ATP hydrolysis but independent of the transport components mediating conventional NLS-dependent nuclear accumulation including importin and probably Ran. In contrast to conventional NLSs such as that of T-ag (see Ref. 6), the Tat-NLS appears to mediate binding to cytoplasmic components (see Ref. 38) in the absence of ATP, as well as conferring passage through the nuclear envelope, and the ability to bind to nuclear components (see Ref. 38) in the presence of ATP. It seems reasonable to propose that the Tat-NLS is recognized by a carrier/receptor protein which mediates nuclear entry and binding to nuclear components, as well as binding to insoluble cytoplasmic structures in the absence of ATP.

At the sequence level, the Tat-NLS is more closely related, especially in terms of the preponderance of positive charge, to the more conventional importin/karyopherin α/β-recognized NLSs of T-ag and bipartite NLSs. That it does not mediate an import pathway comparable to that mediated by these types of NLS, however, is indicated by the fact that: 1) the Tat-NLS does not require cytosolic factors to function and is not recognized by importin/karyopherin α and/or β; 2) nuclear import conferred by the Tat-NLS cannot be competed by excess T-ag NLS peptide; 3) the Tat-NLS confers binding to nuclear components, in contrast to the NLSs of T-ag and Rb (6) (see also below); and 4) regardless of the intactness of the nuclear envelope, the Tat-NLS confers cytoplasmic retention in the absence of ATP hydrolysis, whereas proteins carrying the T-ag- and Rb-NLSs equilibrate between nuclear and cytoplasmic compartments if there is no intact nuclear envelope, irrespective of the presence of ATP (6).

In contrast to conventional NLSs and that of Tat, the M9-NLS of hnRNP A1 is largely hydrophobic. The KNS-NLS of hnRNP K (YDRRGRPGDRYDGMVGFSADETWDSAIDTWS-PSEWQMAY[361]) is rich in serine/threonine, acidic amino acids, and aromatic/small chain hydrophobic amino acids, as well as containing a few basic residues (bold type) toward its amino terminus. Removal of amino acids 359–361 reduces nuclear targeting (17), indicating that these basic residues alone are not the key elements of the NLS, and that the KNS-NLS is fundamentally different from that of Tat. Both M9 and KNS confer specific nuclear export under certain conditions (15–17) and hence are perhaps more appropriately named shuttling sequences rather than NLSs, but there is no evidence that the Tat-NLS can confer nuclear export.[2] While M9-dependent nuclear import requires the cytosolic factors transportin (15, 16, 39) and Ran (see Ref. 39), KNS-mediated nuclear import appears to require only ATP hydrolysis (17), thus resembling the Tat-NLS in this respect. However, there is no evidence for cytoplasmic retention in the absence of ATP hydrolysis or in the presence of nucleotide analogs in the case of the KNS-NLS (see Ref. 17), making it clearly different from that mediated by the Tat-NLS. The nature of the Tat-NLS and its conferred nuclear import properties are accordingly quite different from those of the hnRNP NLSs.

In conclusion, the results here demonstrate that while the Tat-NLS can function as a nuclear entry signal since it is able to target the 476-kDa heterologous protein β-galactosidase through the NPC, it has a unique property in that it confers

accumulation through binding to nuclear components. No such properties have been reported either for the conventional basic NLSs or for the M9- or KNS-NLSs. Based on the homologies between the NLSs of Tat, Rex, and Rev (see introduction) and the fact that they can substitute functionally for one another in various assays (40–42), future work within this laboratory will be directed toward determining whether the Rex and Rev NLSs confer nuclear transport through a pathway similar to that conferred by Tat.

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